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Comparative Analysis of Two Vector Systems in mRNA Vaccine Development

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ABSTRACT

The present study aimed to produce an mRNA vaccine and analyze two types of vectors. According to this study, the Food and Drug Administration has fully approved the mRNA vaccine as the most effective vaccine against the 2019 coronavirus sickness. The lack of anti-vector immunity, the infrequent occurrence of insertional mutagenesis associated with it, and its therapeutic efficacy all contribute to this approval. In addition, it has an edge over traditional DNA vaccines due to its quick production method. A review was conducted based on the available online literature. The procedures used to generate mRNA vaccine in vitro are described in detail in this review. A plasmid containing the super folder green fluorescent protein and the receptor-binding domain present in the coronavirus spike protein is used as an example. A notice for further information on the use of a viral vector in the production of these vaccines is also included.

Keywords: ARCA, Anti-vector immunity, COVID-19, Insertional mutagenesis, Phosphatase, Transfection

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Introduction

The two methods for producing the messenger ribonucleic acid (mRNA) vaccine in vitro are either template-directed modification, also referred to as co-transcriptional modification, or enzymatic modification, which involves altering the poly (A) tail and mRNA cap post-transcriptionally.
[1-4]. During the post-transcriptional modification of mRNA, primarily in eukaryotes, the creation of the cap-0 structures is catalyzed by enzymes like guanylyl transferases. Together with RNA triphosphatase, RNA guanine-N7-methyltransferase, and RNA guanylyltransferase perform the sequential enzymatic processes necessary to create cap-0. The template-directed approach is a better option because of the complexity of the enzymatic capping procedure, which restricts its frequent use and may not be cost-effective [2-4]. Vaccination, utilizing as an example a plasmid containing Super folder green fluorescent protein (SFGFP) is attached to the receptor-binding domain (RBD) of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [5-9]. The present study aimed to produce an mRNA vaccine and analyze two types of vectors. A diagram is used to depict the process's overview **(Figure 1)**.

Materials and Methods

Several published works were searched online, and applicable literature was used to review the creation of mRNA vaccines using vectors and the in vitro transcription process.

Results and Discussion

According to the PCR mixture's components **(Table 1)**, the PCR cycling methodology **(Table 2)**, the NTP/cap analog mixture's composition **(Table 3)**, the in vitro transcription (IVT) reaction mixture's composition **(Table 4)**, and the roles of the components utilized to produce IVT mRNA, several results evaluated from the literature are shown in **Tables 1-4**.

Generation of IVT mRNA vaccine: A plasmid vector perspective

Using a plasmid vector to create the IVT mRNA vaccine entails first creating a large amount of DNA and then turning it into an mRNA. The first phases in the DNA production process are the extraction of external DNA templates, the amplification of the linearized DNA, its purification and testing, and finally its transcription into mRNA. Notably, upon generation, DNA is purified using purification kits and subjected to DNA gel electrophoresis for quality control testing.

Additionally, the generated mRNA is purified using purification kits, and RNA gel electrophoresis is used for quality control testing. Fluorescence microscopy and flow cytometry are used for analysis once they are transfected into the mammalian cells. The sections that follow provide clarification on these procedures.

Transformation of cells and extraction of exogenous DNA

Using plasmids, cells can be transformed. During transformation, the super optimum broth with catabolite repression (SOC) medium is used to introduce the plasmids containing the exogenous DNA into the *E. coli*. The LB-Agar is used to cultivate the *E. coli* for the entire night. The gene encoding the spike protein of the severe acute respiratory syndrome coronavirus 2's receptor-binding domain (RBD) (SARS-CoV-2) is encoded in ampicillin-resistant plasmid DNA (pDNA), which is extracted using ampicillin. The SFGFP, which functioned as the selective marker, is also present [5-7]. The plasmid purification kits are used to purify the plasmids [5].

Amplification of linearized DNA template (plasmid DNA)

Plasmids are buffed by a process called linearization. Throughout the transcription process, linearisation aids in achieving a clear stop and precise length. Because plasmid DNA (pDNA) is wild and contains contaminants, this method is necessary. These contaminants may typically be remnants of the mixing of three different types of pDNA (supercoiled, circular, or linear) and bacterial genomic DNA in varying amounts. Linearisation is also required since circular plasmids' RNA polymerases are catalytic [3, 10-12]. A bacteriophage promoter, a distinct blunt-ended restriction site, an open reading frame (ORF), and optionally a poly(d(A/T)) sequence transcribed into poly(A) are all included in the majority of pDNA templates used for in vitro transcription. Using a restriction enzyme, the plasmid was linearised at this specific blunt-ended restriction site to guarantee a clear stop and defined lengths in the transcription process. With its unique phage DNA-dependent RNA polymerase, the T7 promoter region is the most frequently utilized among the well-known promoter regions T3, T7, or SP6 [13-16].

Using the components of the Polymerase Chain procedure (PCR) procedure **(Table 1)**, Avci-Adali *et al*. [5] showed how the DNA template might be linearized and amplified. A PCR cycling pool is used to pass these components **(Table 2)**.

Table 1. Constituents of the PCR mixture

Constituent	Final concentration	Volume (µL)
Reverse Primer	0.7 µM	7
Forward Primer	0.7 µM	7
5x PCR Buffer	1x	20
5x Q-Solution	1x	20
DNA (Plasmid)	50 ng / 100µL	Variable
DNA Polymerase (2.5 U/µl)	2.5 U	1
Nuclease-free water		Variable
Total volume		100

Table 1 shows the quantity of the constituents of the PCR mixture [5].

Table 2. PCR cycling protocol

Event	Cycle number	Time	Temperature (°C)	Aim of event
Initial denaturation step	1	3-7min	90-100	The dsDNA is denatured into single strands to enhance amplification.
3-step cycling:	2-25			The hydrogen bonds between complementary bases are denatured.
-Denaturation		40-50 sec	90-100	
-Annealing		1-3 min	50-60	The primers are hybridized to the templates.
-Extension		1-3 min	70-75	The hybrid is extended via Polymerase.
Final extension step	26	10-15min	70-75	The primer sequences are extended from the 3' end of the primer.
End of cycling		Indefinite	2-4	The cells are preserved.

Table 2 shows the events in the PCR cycling protocol and their aims [5, 13-16].

The in vitro transcription (IVT)/ DNase reaction

5-Methylcytidine-5'-triphosphate (Me-CTP), pseudouridine-5'-triphosphate (Pseudo-UTP), and the 3′-O-Me-m7G(5')ppp(5')G RNA cap structure analogue, or ARCA, are all produced by trilink biotechnologies. The concentration of nucleoside triphosphate and the template DNA determine the yield and amount of full-length mRNA transcript that is generated. The length of CDS and the quantity of PCR product utilized for IVT dictate the amount of synthesized mRNA. To determine the ideal incubation period, a time-course experiment could be conducted [5]. With the 7-methylated G (m7G) acting as the terminal residue, the proper transcripts are created using 5′-m7G carrying ARCA during template-directed co-transcriptional capping of mRNA. This enables their enhanced translation and proper orientation [17-19]. mRNA vaccines' in vivo immunogenicity is intended to be avoided by the modified nucleoside triphosphate. Some mRNA vaccines may not include adjuvants because of the necessity to avoid this immunogenicity.

Table 3. Constituents of the NTP/cap an1alog mixture

Constituents	Stock concentration (mM)	Final concentration (mM)	Amount (µL)
ARCA	10	2.5	10
GTP	75	1.875	1
ATP	75	7.5	4
Pseudo-UTP	100	7.5	3
Me-CTP	100	7.5	3
Total volume			23

The NTP/cap analog mixture's volumes and concentrations are displayed in **Table 3** [5]. RNase inhibitor, NTP/cap analog mixture, nuclease-free water, reaction buffer (10x), PCR product, and the T7 RNA polymerase enzyme mix (10x) are the materials that make up the in vitro transcription (IVT) reaction mixture **(Table 4)**. Each of these components performs a specific role, as shown in **Table 5**. Since the enzyme DNase selectively breaks down DNA, the DNase treatment aids in the removal of genomic DNA, which is now a contaminant in the RNA sample [5, 20, 21]. Purification kits can be used to purify the PCR results.

Table 4. Constituents of *in vitro* transcription (IVT) reaction mixture

Constituents	Final concentration	Volume (µL)
PCR product	1 µg	Variable
NTP/cap analog mixture		23
Reaction buffer	1x	4
RNase Inhibitor	40 U	1
T7 RNA polymerase enzyme mix	1x	4

Nuclease-free water	Variable
Total volume	40

Table 4 shows the constituents of the IVT reaction mixture with their respective concentration and volume [5].

Purification and quality control of DNA and synthetic mRNA

DNA purification kits can be used to purify DNA, and DNA gel electrophoresis can be used to assess the purification's quality. Similarly, after the mRNA is purified using the RNA purification kit, the quality of the mRNA transcript generated can be determined using RNA gel electrophoresis. The mRNA transcript is then treated with phosphatases to dephosphorylate it [5].

Transfection and analysis of mRNA containing HEK 293 cells

The aliquots of DMEM (200 µl) and Matrigel® (250 µl) are heated to -4 °C [22, 23], which is the optimal temperature for the miscibility of the two substances. After that, the 450 µl homogeneous solution is moved into 40 ml of DMEM, combined, and then added to the 12-well plate (1 ml/well).

Transfection is the introduction of exogenous materials into a mammalian cell. Following that, the plate is incubated for two hours to a maximum of 7 days. The human embryonic kidney 293 (HEK 293) cells are grown in three of these 12 wells to a confluency of 60% to 80%. Within 48 hours, this growth is carried out at 37 °C with 5% CO2. Without the cells achieving 100% confluency, the range of 60-80% confluency allows for additional downstream processes like flow cytometry and fluorescence microscopy. This is because contact inhibition, which causes the cells to wither, would occur at 100% confluency. Until the operation is finished, the wasted DMEM is replaced every day. Trypsin/EDTA (0.5 ml) is added to the spent DMEM, and the cells are incubated for a maximum of five minutes at 37 °C with 5% CO2 to allow for cell separation. The detached cells are then washed with 1 milliliter of Dulbecco's Phosphate-Buffered Saline during this period. After that, the cells are cultured in a transfection solution containing the mRNA. For four hours, 0.5 ml of the cationic lipid transfection reagent is employed at 37 °C with 5% CO2. After mRNA has been transfected into the cells, fluorescence microscopy is performed. The purpose of fluorescent microscopy is to view cellular structures and investigate protein expression and post-translational changes [5].

DMEM (1 ml) is used to incubate the cells overnight following fluorescence microscopy. Trypsin/EDTA (0.5 ml) is used to separate the cells from the 12-well plate following incubation. The cells are then resuspended in DMEM (10 ml) containing ROCK inhibitor (2 µL/10 ml) after being cleaned in DPBS (1 ml). The resuspended cells are maintained in a flow cytometer, and the fluorescent intensity of the cells—which is based on their size, shape, and complexity—is used to calculate the proportion of the cells expressing SFGFP [5].

Table 5. Critical components needed for the generation of mRNA via the IVT process

Name	Functions
Ampicillin	Ampicillin inhibits the growth of *E. coli*, which has a peptidoglycan cell wall. Therefore, ampicillin-resistant plasmids grow more easily [24].
Transfection solution	The mRNA is transported into the cytosol with the aid of the transfection mixture, such as a cationic lipid transfection reagent. By giving the mRNA a polarity akin to that of the cell's lipid bilayer, this reagent facilitates the mRNA's passage through the cell (plasma membrane).
SOC medium	The SOC medium contains glucose, which helps induce a quick adaptation and rapid *in vitro* growth of *E. coli* via the glucose effect phenomenon [25].
Receptor binding domain (RBD)	RBD is found in the tip of the spike proteins of Coronaviruses. The virus triggers an immune response in humans when it attaches to their ACE 2 receptors via these spikes [4].
Luria-bertani agar (LB-Agar)	Pure plasmids of transformed bacterial cells (*E. coli*) can grow more easily on LB-Agar.
SFGFP	SFGFP is a fusion tag for purifying, quantifying, and monitoring proteins in a flow cytometer [5, 7, 26].

DPBS	Preserving an ideal pH, DPBS, or physiological saline, helps to preserve the cells. It also aids in reversing trypsin/EDTA's hydrolyzing actions.
Restriction enzyme	To guarantee transcripts with specific lengths, the DNA template must be linearised using a restriction enzyme. Additionally, it facilitates the donor sequence's unidirectional orientation [5, 11, 27].
RNase inhibitor	RNase inhibitor annuls ribonucleolytic activity [28-30].
T7 RNA polymerase	T7 RNA polymerase is a T7 template-specific polymerase that transcribes the linearized DNA template into mRNA using the nucleoside triphosphates.
Matrigel	The Matrigel forms the basement membrane needed to support the mammalian (culture) cells. It serves as a surrogate extracellular matrix, which forms the micro-environment for growing and studying the culture cells [22, 23].
Nuclease-free water	Nucleases denature the phosphodiester bonds (strands) of nucleic acids. The nuclease-free water is used to supplement the final volume of the reaction mixture to prevent this effect [31, 32].
DNA polymerase	DNA Polymerase speeds up the synthesis of new strands (daughter strands), which are complementary to the strands of the DNA template contained in the reaction mixture found in the PCR [13-15].
Poly T-tail	Poly T-tail aids efficient translation in the cells; hence, it is needed as a precursor for mRNA. It is transcribed into a poly-A tail needed to transport mRNA molecules [1, 11, 33, 34].
Plasmid DNA	Plasmid DNA is extrachromosomal DNA. It is malleable, self-replicating, and adaptation-efficient. It contains only the coding DNA sequence (exons) needed to be transcribed in bacteria [1-3, 35].
DMEM	The DMEM, a nutrient medium, provides nutrients for the mammalian cells. It contains the minimum essential nutrients for the growth of cells.
PCR buffer	Qiagen posits that PCR buffer contains Factor SB to abrogate the degradation of primers and templates during PCR setup, improving the sensitivity and reliability of high-fidelity PCR.
Q-Solution	According to Qiagen, Q-Solution regulates the melting characteristics of nucleic acids. It improves the efficiency of the PCR. Efficiency may be attenuated by templates with a high GC content or a high degree of secondary structures.
Reverse primer and forward primer	They both flank the target DNA region for amplification. The forward primer (antisense strand) tags the start codon of the template DNA. Reverse primer (sense strand) tags the stop codon on the complementary strand of the DNA [36].
Poly-A polymerase	Poly-A polymerase catalyzes the incorporation of poly(A) tail [1].
Transferases (Guanylyl transferase and 2′-O-methyltransferase)	Guanylyl transferase and 2′-O-methyltransferase aid enzymatic capping. They confer cap 0 (N7MeGpppN) or cap 1 (N7MeGpppN2′-OMe) structures, respectively. Cap 1 structures modify the mRNA and make it not recognized as foreign by the innate immune system [1-3].
HEK 293 cells	The HEK 293 cells are mammalian cells used for *in vitro* studies. They are generally preferred due to their rapid growth.
DNase	DNase eliminates the genomic DNA from the RNA samples [20, 21].
The reaction buffer contains Tris-Hcl and KCl, and sometimes MgCl2	Tris-HCl enhances the permeability of the cell membrane. KCl maintains the integrity of the mitochondria, intracellular tonicity, and biosynthesis of nucleic acids. MgCl2 serves as a support for the enzymes, which are biological catalysts [37, 38].
Anti-Reverse Cap Analog (3´-O-Me-m7G(5')ppp(5')G)	The first nucleotide of the transcript contains an analog of the mRNA cap structure called Anti-Reverse Cap Analogue, or ARCA [1, 2]. By preventing the formation of phosphodiester linkages at this location, the 3´-O-methyl alteration guarantees that mRNA

	capping is carried out in the proper orientation [1-3]. As a result, mRNA vaccines have improved transcription, translation, and stability [17].
Modified nucleosides (Me-CTP and Pseudo-UTP)	Modified nucleosides such as CTP and Pseudo-UTP substitute the immunogenic native forms of cytidine and uridine, respectively, leading to increased translation, efficient base stacking, improved nuclease stability, and decreased *in vivo* immunogenicity [1-3, 19].
ATP and GTP	ATP and GTP are the molecular precursors of the mRNA.
Trypsin/EDTA solution	Trypsin optimizes the detachment of the cells from culture plates via hydrolysis. The ethylenediaminetetraacetic acid (EDTA) is an ion-chelator. It keeps the cells in a life-like form during this detachment and enhances hydrolysis [39-43].
Antarctic phosphatases and inorganic pyrophosphatases	The production of significant quantities of phosphates as byproducts during the creation of proteins and nucleic acids can interfere with the completion of these vital physiological processes. To control and lower the cellular content of these phosphates, phosphatases are essential [43-46]. To control the excess of phosphorothioate, phosphorothiolate, imidiphosphate, and boranophosphate that may be generated by the usage of chemically modified cap analogs, phosphatase treatment may be required during the synthesis of IVT mRNA [2].
Rho-kinase (ROCK) Inhibitor	The cell is fixed by the ROCK inhibitor. Through the regulation of stress fibers and focal adhesions, it contributes to the preservation of the cell's viability and shape. As a result, this restricts cell differentiation and autolysis [47, 48].

Table 5 summarizes the functions of the essential components needed in an *in vitro* generation of mRNA vaccine and its analysis using cell culture.

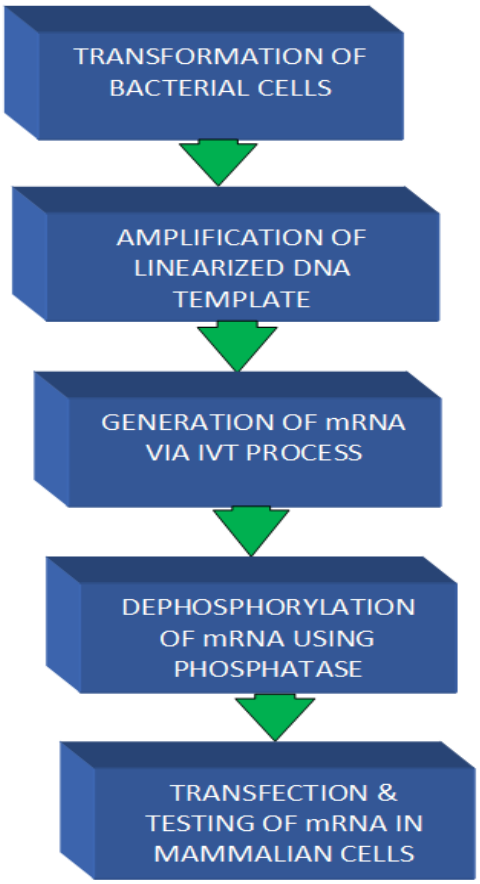

Figure 1. The significant steps in the generation of mRNA vaccine using a plasmid vector.

Purification kits are used to remove the plasmids from the transformed *E. coli* and purify them. The DNA gel electrophoresis is used to test the linearised, amplified, and purified template DNA. RNA gel electrophoresis is used to create, purify, and assess the quality of the IVT mRNA. Phosphatases are used to treat, purify, and transfect the IVT mRNA into the cells. Fluorescence microscopy and flow cytometry are used to examine the cells.

Generation of IVT mRNA vaccine: A viral vector perspective

While the traditional method of creating an IVT mRNA vaccine is to use plasmid vectors as the starting material **(Figure 1)**, viral vectors can also be used in a similar step-by-step fashion. The distinction is that viral vectors are utilized as the beginning material in this case, whereas plasmid vectors are used in the traditional method. Viral vector-based IVT mRNA can be roughly categorized into non-replicating and self-amplifying vaccines, and certain vaccines are created using viral vectors that can amplify minute amounts of the protein, which may result in another difference in their intracytoplasmic effects. The latter, which has a replicase mechanism, is known as self-amplifying mRNA (SAM) vaccines because it replicates, while the former is known as non-replicating mRNA (NRM) vaccines since it does not [1-3, 11]. The mRNA vaccinations, often referred to as trans-amplifying RNA vaccines, were made possible by the modification of the SAM vaccines, also known as the replicon RNA vaccines [49, 50].

RNA viruses are frequently used as vectors in vaccines produced using viral vectors, such as the NRM and SAM. These RNA viral vectors have demonstrated encouraging outcomes against a variety of cancer types and highly contagious diseases. The way the NRM vaccines are delivered allows for further classification: Dendritic cells can be injected directly into human body sites or infused ex vivo [1]. A 5′ and 3′ untranslated region (UTR), ORF, a cap structure, and a 3′ poly(A) tail are shared by both. What set SAM vaccines apart was the addition of genetic replication machinery derived from positive sense single-stranded mRNA viruses, primarily from Flaviviruses like the Kunjin virus and Alphaviruses like the Sindbis virus and Semliki Forest virus. The gene of interest (GoI) often replaces the ORF that codes for the structural proteins of the virus, and the virus's RNA-dependent RNA polymerase is employed to aid in the amplification of the replicon construct in the cytosol [1, 11].

The SAM vaccines do not require an adjuvant because they contain the replicase machinery. This is because, once in the cytoplasm, the replicase machinery increases the dosage of these vaccinations. Therefore, a small dose might be intracellularly amplified to a big amount, which would be adequate to provide the required long-lasting immunity. Since the NRM vaccines, which are mostly utilized as anti-cancer vaccines, lack replicase machinery, they need adjuvants such as key-hole limpet hemocyanin, which is why the SAM vaccines are more effective as prophylactics for infectious diseases [1, 51].

The SAM mRNA vaccine's usage as a preventative measure against infectious diseases is its defining feature. An application whose clinical effectiveness is based on the poly-A-tail qualities, the coding sequence change, the UTR length, structure, and regulatory elements, the efficiency and structure of the 5′ cap, and the purity [1, 3]. The SAM vaccinations used to prevent COVID-19 are not linked to anti-vector immunity, which is a form of protection against the Adenovirus-based viral vectors that people who have already been exposed to this virus exhibit [52, 53]. The effectiveness of these vaccines may be diminished by this anti-vector immunity. A process known as “insertional mutagenesis” occurs when exogenous nucleic acid sequences integrate into the host cell's genome, deregulating genes around the insertion site and occasionally disrupting cellular phenotype [54]. Insertional mutagenesis is rarely linked to the mRNA vaccination. Additionally, because of the strict purification and refinement procedures used in its creation, the mRNA vaccine is not contagious. Additionally, these purification methods avoid the need for antibiotics like ampicillin. The most significant drawback of the mRNA vaccine, despite its enormous advancements, is its inability to contend with viruses with a high capacity for mutation because it is mostly made up of modified nucleosides, a kind of synthetic nucleotide. Changes in the nucleotide sequence that result from mutation affect the strains. While recombination may cause significant genetic changes that render vaccinations ineffective, subtle genetic alterations in certain viruses may result in mutations that reduce the vaccine's efficacy [55-59]. The propensity of coronaviruses to exhibit trans-species mobility and adaptation may always affect the effectiveness of the mRNA vaccination according to the host species, time of year, and geographic location [56-60]. A common scenario in the continuing epidemic is that the vaccine's efficacy decreases when new strains of SARS-CoV-2 arise. As reported by Consumer News and Business Channel, Pfizer claims that the South African strain of SARS-CoV-2 (B.1.351) outperformed the effectiveness of the mRNA vaccine in South Africa by almost 70% [60].

Conclusion

Cell cultures or laboratory animals are used in the arduous process of producing a powerful vaccine to assess the final product's quality. This intensive method necessitates extensive knowledge and experience in clinical immunology and related molecular techniques. Hence, to increase the manufacture of these vaccinations, the governments of different nations and their public health organizations might enlist the help of experts in the field. Despite the vaccinations' variable effectiveness among individuals, which is partially due to pharmacogenomics, this would significantly help stop the pandemic.

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